On page 75, line 9, delete "12301 Parklawn Drive, Rockville, Maryland 20852" and insert --10801 University Boulevard, Manassas, Virginia 20110-2209--.

Q10

REMARKS

Attached herewith as Exhibit A are Marked-Up copies of the amended pages, as Exhibit B are Substitute Sheets of the amended pages, and as Exhibit C the sequence listing in paper form.

Applicants have canceled claims 1-30 and replaced them with new claims 31-36. Therefore, Applicants will only consider new claims 31-36 for the purpose of calculating the filing fees associated with the Continuation Application filed concurrently herewith.

The computer readable form of the "Sequence Listing" in this application, filed herewith, is identical with that filed in USSN 09/167,874, filed October 7, 1998. In accordance with 37 C.F.R. § 1.821(e), please use the first-filed computer readable form filed in that application as the computer readable form for the instant application. It is understood that the Patent and Trademark Office will make the necessary change in application number and filing date for the computer readable form that will be used for the instant application. A paper copy of the Sequence Listing is included as Exhibit C.

I hereby state that the content of the paper readable and computer readable copy of the Sequence Listing submitted herewith and referred to herein in accordance with 37 C.F.R. § 1.821(g), contain no new subject matter.

Applicants direct the subject Sequence Listing submitted herewith be added to the specification.

No fee is deemed necessary in connection with filing this Preliminary Amendment. However, if any fee is deemed necessary, authorization is hereby given to charge the amount of any such fee to Deposit Account No. 18-0650.

Respectfully submitted,

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DORSAL TISSUE AFFECTING FACTOR AND COMPOSITIONS

United States Patent application Serial No. 09/167,874 filed on October 7, 1998, now allowed, which is a continuation of United States Patent Application Serial No. 08/489,721 filed on June 7, 1995, now issued as Patent No. 5,821,124, which is a continuation of

This application is a continuation of ~~copending~~ United States Patent application Serial No. 08/392,935 filed on March 3, 1995, now issued as Patent No. 5,843,775 which is the U.S. National Stage of PCT/US93/08326 filed September 2, 1993, now abandoned, which is a continuation-in-part of U.S. Serial No. 07/957,401 filed on October 6, 1992, now abandoned, which is a continuation-in-part of United States Patent Application Serial No. 07/950,410 filed on September 23, 1992, now abandoned, which is a continuation-in-part of United States Patent Application Serial No. 07/939,954 filed on September 3, 1992, now abandoned.

Field of the Invention

The invention generally relates to growth factors and neurotrophic factors, and more particularly to a soluble growth factor with dorsal growth inducing activity, to complexes including the factor, and to DNA or RNA coding sequences for the factor.

This invention was made, in part, with government support under Grant Contract No. ROI-GM-42341, awarded by the National Institutes of Health. The government has certain rights in this invention.

Background of the Invention

Growth factors are substances, such as polypeptide hormones, which affect the growth of defined populations of animal cells in vivo or in vitro, but which are not nutrient substances. Proteins involved in the growth and differentiation of tissues may promote or inhibit growth, and promote or inhibit differentiation, and thus the general term "growth factor" includes cytokines and trophic factors. Among growth, or neurotrophic factors presently known are those that can be classified into the insulin family [insulin, insulin-like growth

MARKED-UP VERSION

1,2: Recombinant noggin produced in *E. coli* and in insect cells respectively, treated with 2 mercaptoethanol before electrophoresis. The slower mobility of noggin from insect cells corresponds to the size increase that would occur due to N-linked glycosylation at the single consensus site. Lanes 2,3: Recombinant noggin produced in *E. coli* and in insect cells respectively, not treated with 2-mercaptoethanol before electrophoresis.

Figure 11. Circular dichroism spectra of recombinant noggin produced in *E. coli* (--), and in insect cells (-).

Figure 12. Ventral marginal zone assay showing induction of muscle actin mRNA after exposure to human noggin (0.01, 0.05, 0.2 µg/ml) produced in baculovirus, a mock transfected culture of baculovirus (0.02, 1 µg/ml) or human noggin produced in *E.coli* (0.1, 0.5, 2, or 10 µg/ml).

Figure 13. Nucleotide sequence (SEQ ID NO:~~25~~ 10) for the mouse noggin gene and deduced amino acid sequence (SEQ ID NO:~~26~~ 11).

Summary of the Invention

In one aspect of the present invention a peptide that can be in substantially purified form is characterized by one or more of the following, highly conserved amino acid sequences:

QMWLWSQTFCPVLY (SEQ ID NO:3);
RFWPRYVKVGSC (SEQ ID NO:4);
SKRSCSVPEGMVCK (SEQ ID NO:5);
LRWRCQRR (SEQ ID NO:6); and,
ISECKCSC (SEQ ID NO:7).

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Peptides of the invention induce dorsal growth in vertebrates and can be prepared in soluble, physiologically active form for a number of therapeutic, clinical, and diagnostic applications.

In a preferred embodiment, human noggin protein as set forth in Figure 1 (SEQ ID NO: 2) is prepared for use in therapeutic, clinical and diagnostic applications.

In another aspect of the present invention an oligonucleotide, such as cDNA, is provided having substantial similarity to (or being the same as) ~~SEQ ID NO:8 (deduced amino acid sequence, SEQ ID NO: 9), SEQ ID NO: 10 (deduced amino acid sequence, SEQ ID NO: 11), or~~ Xenopus noggin, partial mouse noggin or SEQ ID NO: 10 SEQ ID NO:1. This oligonucleotide can be single or double stranded, be formed of DNA or RNA bases, and can be in the antisense direction with respect to ~~SEQ ID NOS: 8, 10 or 1. SEQ ID NO: 8, SEQ ID NO: 10 and~~ Xenopus noggin, partial mouse noggin, SEQ ID NO: 1 or SEQ ID NO: 10. Xenopus noggin, partial mouse noggin, and SEQ ID NO: 10 SEQ ID NO:1 each code for a functional polypeptide that we have designated "noggin," which is capable of inducing dorsal development in vertebrates when expressed.

Noggin or fragments thereof (which also may be synthesized by in vitro methods) may be fused (by recombinant expression or in vitro covalent methods) to an immunogenic polypeptide and this, in turn, may be used to immunize an animal in order to raise antibodies against a noggin epitope. Anti-noggin is recoverable from the serum of immunized animals. Alternatively, monoclonal antibodies may be prepared from cells to the immunized animal in conventional fashion. Antibodies identified by routine screening will bind to noggin but will not substantially cross-react with "wnt" or other growth factors. Immobilized anti-noggin antibodies are useful particularly in the diagnosis (in vitro or in vivo) or purification of noggin.

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0.15 M NaCl/0.015 M sodium citrate/0.1% NaDodSo4 at 50°C, or (2) use during hybridization a denaturing agent such as formamide, for example, 50% (vol/vol) formamide with 0.1% bovine serum albumin/0.1%Ficoll/0.1% polyvinylpyrrolidone/50 mM sodium phosphate buffer at pH 6.5 with 750 mM NaCl, 75 mM sodium citrate at 42°C.

By "substantial similarity," when we are referring to a nucleotide sequence, is meant cross hybridization of sequences under conditions of moderate stringency using a probe greater than 100 nucleotides long at 30°C in a standard buffer (Wahl et al., PNAS,76, 3683) and washes at 37°C in 300 mM NaCl, 30 mM sodium citrate, 0.2% SDS at pH 7. Alternatively, one is able to isolate, by polymerase chain reaction, a fragment of DNA coding for noggin or noggin family members when using primers of degenerate sequence that encode those SEQ ID NOS:3-7.

By "substantial similarity" when reference is made to proteins is that noggin from different species, or noggin family members within a species, will preserve the positions of cysteine residues in at least 80% of positions throughout the protein. Like the neurotrophin family, the sequence of the mature form of noggin and noggin related polypeptides will be identical in at least 40% of positions. Substantial similarity at the protein level includes an ability of a subject protein to compete with noggin for binding to receptors and some (but not all) monoclonal antibodies raised against noggin epitopes.

The cloned cDNA for noggin (derived from frog) is designated herein as ~~SEQ ID NO: 8,~~ Xenopus noggin partial sequence from mouse ~~as SEQ ID NO: 10 or~~ is designated herein as partial mouse noggin, the full sequence of mouse noggin is designated herein as shown in Figure 13 (SEQ ID

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10, and the
NO: ~~25);~~ ~~The~~ human sequence is designated herein as SEQ ID NO: 1. We have used RNA transcripts from the Xenopus noggin ~~SEQ ID NO: 8~~ clone to rescue embryos and return them to substantially normal development when the noggin RNA is injected into ventralized embryos. In high doses this results in excessive head development and it is for this reason we named the protein "noggin." In northern blot analysis the noggin cDNA hybridizes to two mRNAs that are expressed both maternally and zygotically.

When using nucleotide sequences coding for part or all of noggin in accordance with this invention, the length of the sequence should be at least sufficient in size to be capable of hybridizing with endogenous mRNA for the vertebrate's own noggin. Typically, sufficient sequence size (for example, for use as diagnostic probes) will be about 15 consecutive bases (DNA or RNA). In some diagnostic and therapeutic applications, one may wish to use nucleotide noggin coding sequences (analogous to all or a portion of Xenopus noggin, partial mouse noggin, ~~SEQ ID NO: 8, SEQ ID NO: 10,~~ SEQ ID NO: 10, ~~25~~ or SEQ ID NO:1) in the anti-sense direction with respect to either Xenopus noggin, partial mouse noggin, SEQ ID NOS: 10 or 1 ~~SEQ ID NOS: 8, 10, 25, or 1~~.

We suggest as a few preferred primers for amplifying noggin from other species (e.g. human):

5' Primer 1 SEQ ID NO: 12

C A A/G A C N T T C/T T G C/T C C N G T N

5' Primer 2 SEQ ID NO: 13

T T C/T T G G C C N C/A G N T A C/T G T N A A A/G G T N G G

5' Primer 3 SEQ ID NO: 14

C C N G A A/G G G N A T G G T N T G

3' Primer 1 SEQ ID NO: 15

C A N C/G T/A A/G C A C/T T T A/G C A C/T T C

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Practice of this invention includes preparation and uses of a diagnostic or therapeutic agent comprising a nucleotide sequence of at least about 15 DNA or RNA bases analogous to all or a portion of either ~~SEQ ID NO: 8, SEQ ID NO: 10, SEQ ID NO: 25~~, Xenopus noggin, partial mouse noggin, SEQ ID NO:10 or SEQ ID NO: 1 or of the nucleic acid sequences contained in bacteriophages, hnogλ-9 or hnogλ-10. That is, noggin preparations are useful as standards in assays for noggin and in competitive-type receptor binding assays when labelled with radioiodine, enzymes, fluorophores, spin labels, and the like. Therapeutic formulations of noggin are prepared for storage by mixing noggin having the desired degree of purity with optional physiologically acceptable carriers, excipients or stabilizers, in the form of lyophilized cake or aqueous solutions. Acceptable carriers, excipients or stabilizers are nontoxic to recipients at the dosages and concentrations employed, and include buffers such as phosphate, citrate, and other organic acids; antioxidants including ascorbic acid; low molecular weight (less than about 10 residues) polypeptides; proteins, such as serum albumin, gelatin or immunoglobulins. Other components can include glycine, blutamine, asparagine, arginine, or lysine; monosaccharides, disaccharides, and other carbohydrates including glucose, mannose, or dextrins; chelating agents such as EDTA; sugar alcohols such as mannitol or sorbitol; salt-forming counterions such as sodium; and/or nonionic surfactants such as Tween, Pluronics or PEG.

Noggin may be used according to the invention as described supra. The concentration of the active ingredient used in the formulation will depend upon the effective dose required and the mode of administration used. The dose used should be sufficient to

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exonuclease III digestion (Henikoff, Meth. Enzymol, 155, 156-165, 1987).

In vitro translation

One-half μg of in vitro synthesized noggin, Xwnt-8, and goosecoid mRNAs were translated in a nuclease treated rabbit reticulocyte lysate (Promega) with added 35S-methionine according to the manufacturer's instructions. The translation products were visualized by SDS polyacrylamide gel electrophoresis (12% gels) followed by fluorography. Noggin protein had the molecular weight predicted by the open reading frame.

RNA Isolation and Analysis

Total RNA was isolated from embryos and oocytes by a small scale protocol as described by Condie and Harland, supra. Dorsal lips were dissected from 30 unfixed stage 10.5 embryos and pooled for total RNA preparation. Samples containing either the total RNA equivalent of 2.5 embryos or approximately 2 μg of poly A+ RNA were analyzed by northern blotting. Random primed DNA probes were prepared from a 1,323 bp fragment of noggin cDNA from the EcoRI site at nucleotide -83 to an EcoRV site that lies in the vector immediately 3' to the end of the cDNA.

RNAse protection assays were done using a protocol as detailed by Melton et al. (Nuc. Acids Res., 12, 7035-7056, 1984) with minor modifications (C. Kintner, Salk Institute, La Jolla, California). A noggin cDNA exonuclease III deletion clone, illustrated by ~~SEQ ID NO: 8~~ Xenopus noggin but having a deletion from the 3' end to nucleotide 383, was used as a template for synthesizing RNA

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Results

Noggin cDNA Encodes a Novel Polypeptide

The 1833 nucleotide sequence of the selected clone is ~~shown by SEQ ID N0: 8~~ and sometimes also referred to as "clone A3." The sequence contains a single long open reading frame encoding a 222 amino acid polypeptide with a predicted molecular weight of 26 kDa. At the amino terminus, the hydrophobic stretch of amino acids suggests that the polypeptide enters the secretory pathway. There is a single potential site for N-linked glycosylation at amino acid 61. Extensive untranslated regions are located both 5' and 3' to the reading frame (593 and 573 bp, respectively). The 3' untranslated region is particularly rich in repeated dA and dT nucleotides, and contains, in addition to a polyadenylation signal sequence located 24 bp upstream from the start of the poly A tail, a second potential polyadenylation sequence 147 bp further upstream.

Xenopus noggin

Sense RNA synthesized from clone A3 with SP6 RNA polymerase was translated in a rabbit reticulocyte lysate system. The 3S-labeled products were fractionated on a 12% SDS-polyacrylamide gel and visualized by fluorography. The major protein product had the expected molecular weight of approximately 26 kDa.

Comparison of the amino acid sequence of the predicted polypeptide to the National Center for Biotechnology Information BLAST network (non-redundant data base) did not identify any similar sequence. Thus, this clone encodes the new type of protein we have named "noggin" which is secreted, and which has dorsal inducing activity in Xenopus.

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EXAMPLE 3

Cloning of the Mouse Noggin Homolog

It is currently impossible to eliminate zygotic noggin transcription from developing Xenopus embryos. In contrast, it should be possible to generate homozygous null mutations in the mouse. We have cloned the partial mouse noggin cDNA (~~SEQ ID NO: 10~~). This is useful to generate mutant mice. In addition to generating the probes and tools to make mutant mice, a comparison of the noggin sequences should be a useful predictor of conserved domains and functions. The C-terminal 80 amino acids are 87% identical between Xenopus noggin and partial mouse noggin ~~SEQ ID NOS: 8 and 10~~.

Mouse noggin was isolated from an embryonic cDNA library by probing with a radiolabelled frog noggin cDNA under conditions of moderate stringency (as defined earlier). Subsequently a genomic clone was isolated by probing a genomic library with the mouse noggin cDNA 15 under conditions of high stringency (as defined, but hybridized at 42° C and washed at 50°C in 15 mM NaCl, 1.5 mM sodium citrate). The full nucleotide sequence of mouse noggin cDNA (SEQ ID NO: ~~25~~ 10) as well as the deduced amino acid sequence (SEQ ID NO: ~~26~~ 11) are shown in Figure 13. There are only two amino acid differences between mouse noggin and human noggin.

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EXAMPLE 4

Cloning of the Human Noggin Homolog

Materials and Methods

Probe preparation

Two oligonucleotides were synthesized based on the mouse noggin sequence (supra). The sequence of the oligonucleotides is noggin 5' : 5' CAG ATG TGG CTG TGG TCA-3' (SEQ ID NO: 18) corresponding to amino acids QMWLWS (SEQ ID NO: 19) and noggin 3' : 5'-GCAGGAACACTTACACTC 3' (SEQ ID NO: 20) corresponding to amino acids ECKCSC (SEQ ID NO: 21) of the mouse noggin protein .

The oligonucleotides were used for PCR amplification of a segment of DNA of 260 nucleotides using as a template a mouse cDNA clone prepared as set forth in Example 3. The amplified fragment had a nucleotide sequence that corresponds to nucleotides 2 through 262 of the partial mouse noggin sequence ~~as set forth in SEQ ID NO: 10~~. After amplification, the PCR reaction was electrophosed in agarose gels, the DNA band of 260 nts purified by Magic PCR (Promega), and used as template for the probe labeling reaction. The probe was labeled using a standard PCR reaction (Perkin-Elmer) on 20 ng of DNA template and 0.2 m Curie of alpha 32P dCTP (Du Pont 3000 Ci/mmol) instead of dCTP. Unincorporated label was separated from the probes on a G50 NICK column (Pharmacia). The excluded volume of the reaction contained a total of 1.8 x108 cpm.

In addition, one degenerated oligonucleotide, named noggin D, corresponding to conserved mouse and Xenopus noggin sequences, was synthesized as follows: Noggin D: 5'- GARGGIATGGTITGYAARCC-

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mixed with 0.5 ml saturated NM538 culture, incubated for 20 min at 37°C and then inoculated into 250 ml LB containing 10 mM Mg SO4, 0.2% maltose. The cultures were incubated until cell lysis (7-8 hr) at 37°C. The phage lysates were used for phage DNA purification by the Qiagen procedure according to the manufacturers recommendations (Qiagen).

Sequencing

Sequencing was performed by using the Applied Biosystems Model 373A automatic sequencer and Applied Biosystems Taq DyeDeoxy™ Terminator Cycle Sequencing Kit.

Results

Filters hybridized to the PCR mouse noggin probes (SEQ ID NOS: 18 and 20) showed two strong signals corresponding to phage plaques named hnogλ–9 and hnogλ–10. These plaques also hybridized to degenerate oligonucleotide probe nogginD (SEQ ID NO: 22) revealed that these clones correspond to the human noggin gene. In addition, two other plaques named hnogλ-5 and hnogλ-7 produced slightly weaker signals when hybridized to the PCR probes. These clones correspond to either human noggin or related gene(s). All of the human DNA inserts can be excised from the vectors using known restriction sites as described in the literature regarding each particular library.

A 1.6 kb SacI fragment from clone hnogλ–9 containing the human noggin gene was subcloned and the nucleotide sequence determined as set forth in Figure 1 (SEQ ID NO: 1). The amino acid sequence for human noggin, as deduced from the nucleotide sequence, is also set

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forth in Figure 1 (SEQ ID NO:2). The gene or cDNA may be expressed in various eukaryotic or prokaryotic expression systems to produce biologically active human noggin protein. It is expected that the human protein will exhibit neurotrophic activity similar to that exhibited by Xenopus noggin protein.

EXAMPLE 5

Tissue Localization of message for human noggin

Materials and Methods

Probe preparation

Probes were prepared as set forth in Example 4. The oligos used are as follows:

SEQ ID NO: ~~23~~ 8:

5' GAC.TCG.AGT.CGA.CAT.CGC.AGA.TGT.GGC.TGT.GGT.CAC

SEQ ID NO: ~~24~~ 9:

5' CCA.AGC.TTC.TAG.AAT.TCG.CAG.GAA.CAC.TTA.CAC.TCG.G

(The underlined sequence represent mouse noggin sequence; the rest of the sequence are tails containing restriction sites for cloning.)

A DNA fragment of approximately 300 bp was obtained by PCR amplification of a mouse cDNA clone prepared as described in Example 3.

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noggin produced in E. coli, in insect cells, and in COS-M5 cells. The antibody does not react with the neurotrophins BDNF, NT-3 and NT-4. Western blotting showed that the antibody detects both reduced and non-reduced protein.

DEPOSIT OF MICROORGANISMS

The following were deposited with the American Type Culture Collection, 10801 University Boulevard, Manassas, Virginia 20110-2209 ~~12301 Parklawn Drive, Rockville, Maryland 20852~~ under the terms of the Budapest Treaty:

		ATCC Accession No.	Date of Deposit
phage	hnogλ-5	75311	9-23-92
phage	hnogλ-7	75309	9-23-92
phage	hnogλ-9	75310	9-23-92
phage	hnogλ-10	75308	9-23-92
hybridoma	RP57-16	CRL 11446	8-25-93

It is to be understood that while the invention has been described above in conjunction with preferred specific embodiments, the description and examples are intended to illustrate and not limit the scope of the invention, which is defined by the scope of the appended claims.

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DORSAL TISSUE AFFECTING FACTOR AND COMPOSITIONS

This application is a continuation of United States Patent application Serial No. 09/167,874 filed on October 7, 1998, now allowed, which is a continuation of United States Patent application Serial No. 08/485,721 filed on June 7, 1995, now issued as Patent No. 5,821,124, which is a continuation of United States Patent application Serial No. 08/392,935 filed on March 3, 1995, now issued as Patent No. 5,843,775, which is the U.S. National Stage of PCT/US93/08326 filed September 2, 1993, now abandoned, which is a continuation-in-part of U.S. Serial No. 07/957,401 filed on October 6, 1992, now abandoned, which is a continuation-in-part of United States Patent Application Serial No. 07/950,410 filed on September 23, 1992, now abandoned, which is a continuation-in-part of United States Patent Application Serial No. 07/939,954 filed on September 3, 1992, now abandoned.

Field of the Invention

The invention generally relates to growth factors and neurotrophic factors, and more particularly to a soluble growth factor with dorsal growth inducing activity, to complexes including the factor, and to DNA or RNA coding sequences for the factor.

This invention was made, in part, with government support under Grant Contract No. ROI-GM-42341, awarded by the National Institutes of Health. The government has certain rights in this invention.

Background of the Invention

Growth factors are substances, such as polypeptide hormones, which affect the growth of defined populations of animal cells in vivo or in vitro, but which are not nutrient substances. Proteins involved in the growth and differentiation of tissues may promote or inhibit growth, and promote or inhibit differentiation, and thus the general term "growth factor" includes cytokines and trophic factors. Among growth, or neurotrophic factors presently known are those that can be classified into the insulin family [insulin, insulin-like growth

1 continued

1,2: Recombinant noggin produced in *E. coli* and in insect cells respectively, treated with 2 mercaptoethanol before electrophoresis. The slower mobility of noggin from insect cells corresponds to the size increase that would occur due to N-linked glycosylation at the single consensus site. Lanes 2,3: Recombinant noggin produced in *E. coli* and in insect cells respectively, not treated with 2-mercaptoethanol before electrophoresis.

Figure 11. Circular dichroism spectra of recombinant noggin produced in *E. coli* (--), and in insect cells (-).

Figure 12. Ventral marginal zone assay showing induction of muscle actin mRNA after exposure to human noggin (0.01, 0.05, 0.2 µg/ml) produced in baculovirus, a mock transfected culture of baculovirus (0.02, 1 µg/ml) or human noggin produced in *E.coli* (0.1, 0.5, 2, or 10 µg/ml).

Figure 13. Nucleotide sequence (SEQ ID NO:10) for the mouse noggin gene and deduced amino acid sequence (SEQ ID NO:11).

Summary of the Invention

In one aspect of the present invention a peptide that can be in substantially purified form is characterized by one or more of the following, highly conserved amino acid sequences:

QMWLWSQTFCPVLY (SEQ ID NO:3);
RFWPRYVKVGSC (SEQ ID NO:4);
SKRSCSVPEGMVCK (SEQ ID NO:5);
LRWRCQRR (SEQ ID NO:6); and,
ISECKCSC (SEQ ID NO:7).

Peptides of the invention induce dorsal growth in vertebrates and can be prepared in soluble, physiologically active form for a number of therapeutic, clinical, and diagnostic applications.

In a preferred embodiment, human noggin protein as set forth in Figure 1 (SEQ ID NO: 2) is prepared for use in therapeutic, clinical and diagnostic applications.

In another aspect of the present invention an oligonucleotide, such as cDNA, is provided having substantial similarity to (or being the same as) Xenopus noggin, partial mouse noggin SEQ ID NO:1 or SEQ ID NO: 10. This oligonucleotide can be single or double stranded, be formed of DNA or RNA bases, and can be in the antisense direction with respect to Xenopus noggin, partial mouse noggin, SEQ ID NO: 1 or SEQ ID NO: 10. Xenopus noggin, partial mouse noggin, SEQ ID NO:1 and SEQ ID NO: 10 each code for a functional polypeptide that we have designated "noggin," which is capable of inducing dorsal development in vertebrates when expressed.

Noggin or fragments thereof (which also may be synthesized by in vitro methods) may be fused (by recombinant expression or in vitro covalent methods) to an immunogenic polypeptide and this, in turn, may be used to immunize an animal in order to raise antibodies against a noggin epitope. Anti-noggin is recoverable from the serum of immunized animals. Alternatively, monoclonal antibodies may be prepared from cells to the immunized animal in conventional fashion. Antibodies identified by routine screening will bind to noggin but will not substantially cross-react with "wnt" or other growth factors. Immobilized anti-noggin antibodies are useful particularly in the diagnosis (in vitro or in vivo) or purification of noggin.

0.15 M NaCl/0.015 M sodium citrate/0.1% NaDodSo4 at 50°C, or (2) use during hybridization a denaturing agent such as formamide, for example, 50% (vol/vol) formamide with 0.1% bovine serum albumin/0.1%Ficoll/0.1% polyvinylpyrrolidone/50 mM sodium phosphate buffer at pH 6.5 with 750 mM NaCl, 75 mM sodium citrate at 42°C.

By "substantial similarity," when we are referring to a nucleotide sequence, is meant cross hybridization of sequences under conditions of moderate stringency using a probe greater than 100 nucleotides long at 30°C in a standard buffer (Wahl et al., PNAS,76, 3683) and washes at 37°C in 300 mM NaCl, 30 mM sodium citrate, 0.2% SDS at pH 7. Alternatively, one is able to isolate, by polymerase chain reaction, a fragment of DNA coding for noggin or noggin family members when using primers of degenerate sequence that encode those SEQ ID NOS:3-7.

By "substantial similarity" when reference is made to proteins is that noggin from different species, or noggin family members within a species, will preserve the positions of cysteine residues in at least 80% of positions throughout the protein. Like the neurotrophin family, the sequence of the mature form of noggin and noggin related polypeptides will be identical in at least 40% of positions. Substantial similarity at the protein level includes an ability of a subject protein to compete with noggin for binding to receptors and some (but not all) monoclonal antibodies raised against noggin epitopes.

The cloned cDNA for noggin (derived from frog) is designated herein as Xenopus noggin partial sequence from mouse is designated herein as partial mouse noggin, the full sequence of mouse noggin as shown in Figure 13 is designated herein as SEQ ID

NO: 10, and the human sequence is designated herein as SEQ ID NO: 1. We have used RNA transcripts from the Xenopus noggin clone to rescue embryos and return them to substantially normal development when the noggin RNA is injected into ventralized embryos. In high doses this results in excessive head development and it is for this reason we named the protein "noggin." In northern blot analysis the noggin cDNA hybridizes to two mRNAs that are expressed both maternally and zygotically.

When using nucleotide sequences coding for part or all of noggin in accordance with this invention, the length of the sequence should be at least sufficient in size to be capable of hybridizing with endogenous mRNA for the vertebrate's own noggin. Typically, sufficient sequence size (for example, for use as diagnostic probes) will be about 15 consecutive bases (DNA or RNA). In some diagnostic and therapeutic applications, one may wish to use nucleotide noggin coding sequences (analogous to all or a portion of Xenopus noggin, partial mouse noggin, SEQ ID NO: 10 or SEQ ID NO:1) in the anti-sense direction with respect to either Xenopus noggin, partial mouse noggin, SEQ ID NOS: 10 or 1.

We suggest as a few preferred primers for amplifying noggin from other species (e.g. human):

5' Primer 1 SEQ ID NO: 12

C A A/G A C N T T C/T T G C/T C C N G T N

5' Primer 2 SEQ ID NO: 13

T T C/T T G G C C N C/A G N T A C/T G T N A A A/G G T N G G

5' Primer 3 SEQ ID NO: 14

C C N G A A/G G G N A T G G T N T G

3' Primer 1 SEQ ID NO: 15

C A N C/G T/A A/G C A C/T T T A/G C A C/T T C

Practice of this invention includes preparation and uses of a diagnostic or therapeutic agent comprising a nucleotide sequence of at least about 15 DNA or RNA bases analogous to all or a portion of either Xenopus noggin, partial mouse noggin, SEQ ID NO: 10, or SEQ ID NO: 1 or of the nucleic acid sequences contained in bacteriophages, hnogλ-9 or hnogλ-10. That is, noggin preparations are useful as standards in assays for noggin and in competitive-type receptor binding assays when labelled with radioiodine, enzymes, fluorophores, spin labels, and the like. Therapeutic formulations of noggin are prepared for storage by mixing noggin having the desired degree of purity with optional physiologically acceptable carriers, excipients or stabilizers, in the form of lyophilized cake or aqueous solutions. Acceptable carriers, excipients or stabilizers are nontoxic to recipients at the dosages and concentrations employed, and include buffers such as phosphate, citrate, and other organic acids; antioxidants including ascorbic acid; low molecular weight (less than about 10 residues) polypeptides; proteins, such as serum albumin, gelatin or immunoglobulins. Other components can include glycine, blutamine, asparagine, arginine, or lysine; monosaccharides, disaccharides, and other carbohydrates including glucose, mannose, or dextrins; chelating agents such as EDTA; sugar alcohols such as mannitol or sorbitol; salt-forming counterions such as sodium; and/or nonionic surfactants such as Tween, Pluronics or PEG.

Noggin may be used according to the invention as described supra. The concentration of the active ingredient used in the formulation will depend upon the effective dose required and the mode of administration used. The dose used should be sufficient to

exonuclease III digestion (Henikoff, Meth. Enzymol, 155, 156-165, 1987).

In vitro translation

One-half μg of in vitro synthesized noggin, Xwnt-8, and goosecoid mRNAs were translated in a nuclease treated rabbit reticulocyte lysate (Promega) with added 35S-methionine according to the manufacturer's instructions. The translation products were visualized by SDS polyacrylamide gel electrophoresis (12% gels) followed by fluorography. Noggin protein had the molecular weight predicted by the open reading frame.

RNA Isolation and Analysis

Total RNA was isolated from embryos and oocytes by a small scale protocol as described by Condie and Harland, supra. Dorsal lips were dissected from 30 unfixed stage 10.5 embryos and pooled for total RNA preparation. Samples containing either the total RNA equivalent of 2.5 embryos or approximately 2 μg of poly A+ RNA were analyzed by northern blotting. Random primed DNA probes were prepared from a 1,323 bp fragment of noggin cDNA from the EcoRI site at nucleotide -83 to an EcoRV site that lies in the vector immediately 3' to the end of the cDNA.

RNAse protection assays were done using a protocol as detailed by Melton et al. (Nuc. Acids Res., 12, 7035-7056, 1984) with minor modifications (C. Kintner, Salk Institute, La Jolla, California). A noggin cDNA exonuclease III deletion clone, illustrated by Xenopus noggin but having a deletion from the 3' end to nucleotide 383, was used as a template for synthesizing RNA

Results

Noggin cDNA Encodes a Novel Polypeptide

The 1833 nucleotide sequence of the selected clone is Xenopus noggin and sometimes also referred to as "clone A3." The sequence contains a single long open reading frame encoding a 222 amino acid polypeptide with a predicted molecular weight of 26 kDa. At the amino terminus, the hydrophobic stretch of amino acids suggests that the polypeptide enters the secretory pathway. There is a single potential site for N-linked glycosylation at amino acid 61. Extensive untranslated regions are located both 5' and 3' to the reading frame (593 and 573 bp, respectively). The 3' untranslated region is particularly rich in repeated dA and dT nucleotides, and contains, in addition to a polyadenylation signal sequence located 24 bp upstream from the start of the poly A tail, a second potential polyadenylation sequence 147 bp further upstream.

Sense RNA synthesized from clone A3 with SP6 RNA polymerase was translated in a rabbit reticulocyte lysate system. The 3S-labeled products were fractionated on a 12% SDS-polyacrylamide gel and visualized by fluorography. The major protein product had the
expected molecular weight of approximately 26 kDa.

Comparison of the amino acid sequence of the predicted polypeptide to the National Center for Biotechnology Information BLAST network (non-redundant data base) did not identify any similar sequence. Thus, this clone encodes the new type of protein we have named "noggin" which is secreted, and which has dorsal inducing activity in Xenopus.

EXAMPLE 3

Cloning of the Mouse Noggin Homolog

It is currently impossible to eliminate zygotic noggin transcription from developing Xenopus embryos. In contrast, it should be possible to generate homozygous null mutations in the mouse. We have cloned the partial mouse noggin cDNA. This is useful to generate mutant mice. In addition to generating the probes and tools to make mutant mice, a comparison of the noggin sequences should be a useful predictor of conserved domains and functions. The C-terminal 80 amino acids are 87% identical between Xenopus noggin and partial mouse noggin.

Mouse noggin was isolated from an embryonic cDNA library by probing with a radiolabelled frog noggin cDNA under conditions of moderate stringency (as defined earlier). Subsequently a genomic clone was isolated by probing a genomic library with the mouse noggin cDNA 15 under conditions of high stringency (as defined, but hybridized at 42° C and washed at 50°C in 15 mM NaCl, 1.5 mM sodium citrate). The full nucleotide sequence of mouse noggin cDNA (SEQ ID NO: 10) as well as the deduced amino acid sequence (SEQ ID NO: 11) are shown in Figure 13. There are only two amino acid differences between mouse noggin and human noggin.

EXAMPLE 4

Cloning of the Human Noggin Homolog

Materials and Methods

Probe preparation

Two oligonucleotides were synthesized based on the mouse noggin sequence (supra). The sequence of the oligonucleotides is noggin 5' : 5' CAG ATG TGG CTG TGG TCA-3' (SEQ ID NO: 18) corresponding to amino acids QMWLWS (SEQ ID NO: 19) and noggin 3' : 5'-GCAGGAACACTTACACTC 3' (SEQ ID NO: 20) corresponding to amino acids ECKCSC (SEQ ID NO: 21) of the mouse noggin protein . .

The oligonucleotides were used for PCR amplification of a segment of DNA of 260 nucleotides using as a template a mouse cDNA clone prepared as set forth in Example 3. The amplified fragment had a nucleotide sequence that corresponds to nucleotides 2 through 262 of the partial mouse noggin sequence. After amplification, the PCR reaction was electrophosed in agarose gels, the DNA band of 260 nts purified by Magic PCR (Promega), and used as template for the probe labeling reaction. The probe was labeled using a standard PCR reaction (Perkin-Elmer) on 20 ng of DNA template and 0.2 m Curie of alpha 32P dCTP (Du Pont 3000 Ci/mmol) instead of dCTP. Unincorporated label was separated from the probes on a G50 NICK column (Pharmacia). The excluded volume of the reaction contained a total of 1.8 x108 cpm.

In addition, one degenerated oligonucleotide, named noggin D, corresponding to conserved mouse and Xenopus noggin sequences, was synthesized as follows: Noggin D: 5'- GARGGIATGGTITGYAARCC-

mixed with 0.5 ml saturated NM538 culture, incubated for 20 min at 37°C and then inoculated into 250 ml LB containing 10 mM Mg SO4, 0.2% maltose. The cultures were incubated until cell lysis (7-8 hr) at 37°C. The phage lysates were used for phage DNA purification by the Qiagen procedure according to the manufacturers recommendations (Qiagen).

Sequencing

Sequencing was performed by using the Applied Biosystems Model 373A automatic sequencer and Applied Biosystems Taq DyeDeoxy™ Terminator Cycle Sequencing Kit.

Results

Filters hybridized to the PCR mouse noggin probes (SEQ ID NOS: 18 and 20) showed two strong signals corresponding to phage plaques named hnogλ–9 and hnogλ–10. These plaques also hybridized to degenerate oligonucleotide probe nogginD (SEQ ID NO: 22) revealed that these clones correspond to the human noggin gene. In addition, two other plaques named hnogλ-5 and hnogλ-7 produced slightly weaker signals when hybridized to the PCR probes. These clones correspond to either human noggin or related gene(s). All of the human DNA inserts can be excised from the vectors using known restriction sites as described in the literature regarding each particular library.

A 1.6 kb SacI fragment from clone hnogλ–9 containing the human noggin gene was subcloned and the nucleotide sequence determined as set forth in Figure 1 (SEQ ID NO: 1). The amino acid sequence for human noggin, as deduced from the nucleotide sequence, is

also set

forth in Figure 1 (SEQ ID NO: 2). The gene or cDNA may be expressed in various eukaryotic or prokaryotic expression systems to produce biologically active human noggin protein. It is expected that the human protein will exhibit neurotrophic activity similar to that exhibited by Xenopus noggin protein.

EXAMPLE 5

Tissue Localization of message for human noggin

Materials and Methods

Probe preparation

Probes were prepared as set forth in Example 4. The oligos used are as follows:

SEQ ID NO: 8:

5' GAC.TCG.AGT.CGA.CAT.CGC.AGA.TGT.GGC.TGT.GGT.CAC

SEQ ID NO: 9:

5' CCA.AGC.TTC.TAG.AAT.TCG.CAG.GAA.CAC.TTA.CAC.TCG.G

(The underlined sequence represent mouse noggin sequence; the rest of the sequence are tails containing restriction sites for cloning.)

A DNA fragment of approximately 300 bp was obtained by PCR amplification of a mouse cDNA clone prepared as described in Example 3.

noggin produced in E. coli, in insect cells, and in COS-M5 cells. The antibody does not react with the neurotrophins BDNF, NT-3 and NT-4. Western blotting showed that the antibody detects both reduced and non-reduced protein.

DEPOSIT OF MICROORGANISMS

The following were deposited with the American Type Culture Collection,10801 University Boulevard, Manassas, Virginia 20110-2209 under the terms of the Budapest Treaty:

		ATCC Accession No.	Date of Deposit
phage	hnogλ-5	75311	9-23-92
phage	hnogλ-7	75309	9-23-92
phage	hnogλ-9	75310	9-23-92
phage	hnogλ-10	75308	9-23-92
hybridoma	RP57-16	CRL 11446	8-25-93

It is to be understood that while the invention has been described above in conjunction with preferred specific embodiments, the description and examples are intended to illustrate and not limit the scope of the invention, which is defined by the scope of the appended claims.